

Discovery of a Small Molecule TLR3 Agonist Adjuvant

Supplementary Materials

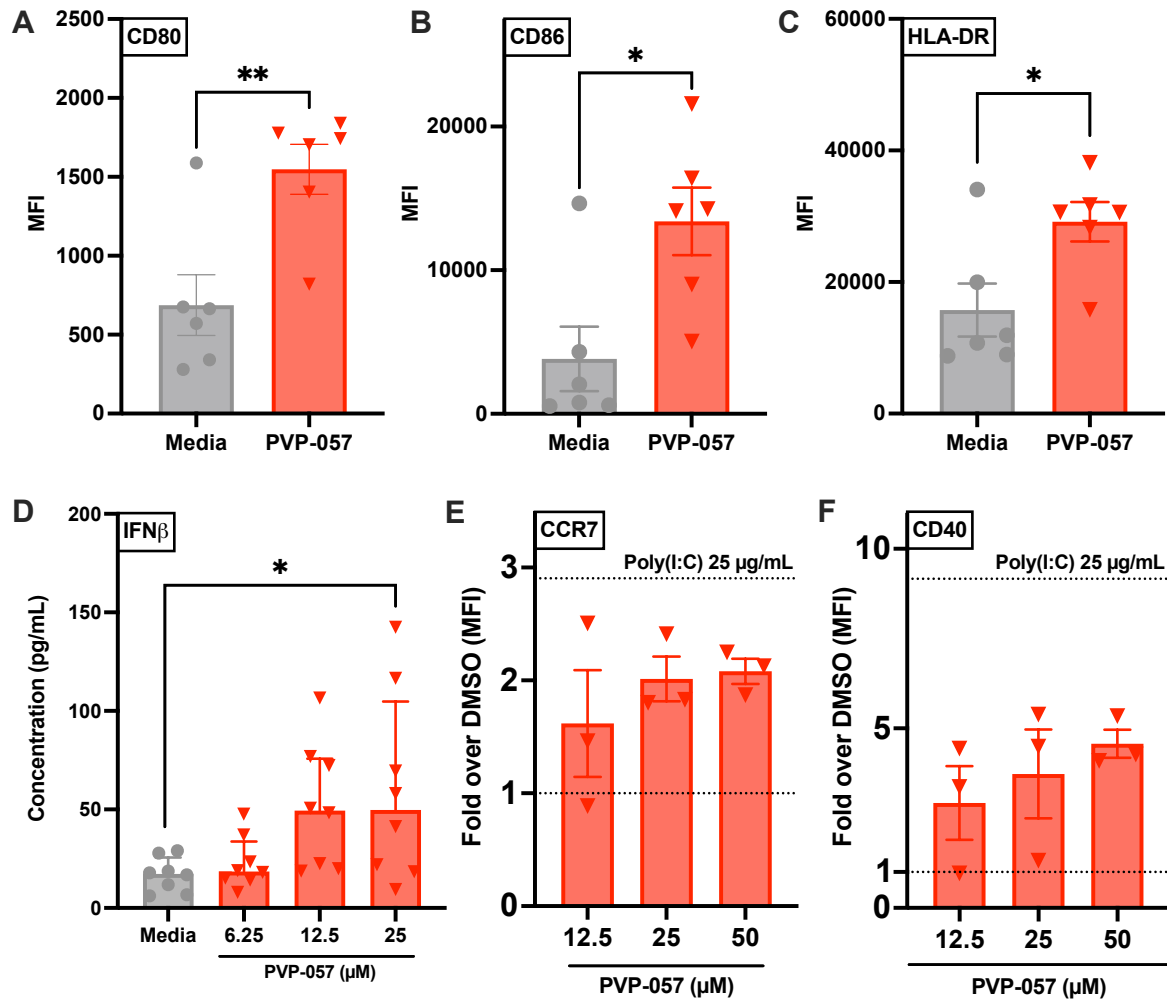
Phase 1: Full HTS for NF- κ B and Adherence

Hit Type	Readout	Hit #	Hit %
Type 1	Luminescence only	884	0.446
Type 2	Luminescence & Fluorescence	139	0.070
Type 3	Fluorescence only	285	0.144
Combined	Combined	1308	0.659

Phase 2: Confirmed hits for NF- κ B and Adherence

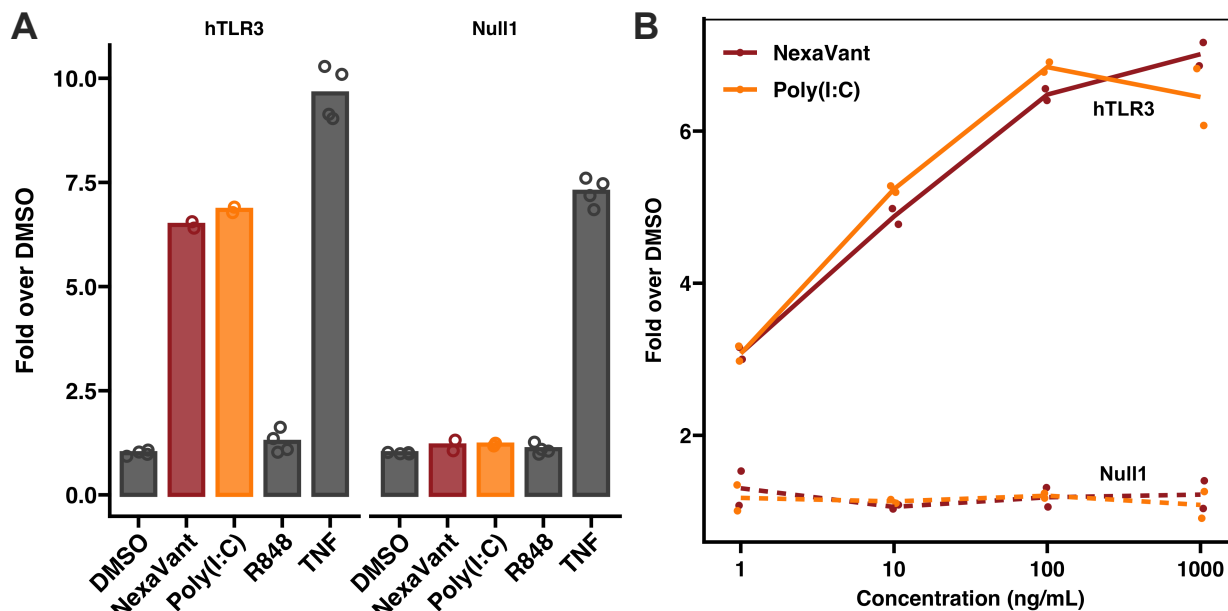
Hit Type	Readout	Hit #	Hit %
Type 1	Luminescence only	105	11.88
Type 2	Luminescence & Fluorescence	24	17.27
Type 3	Fluorescence only	38	13.33
Combined	Combined	167	12.77

Supplementary Table 1. Overview of High Throughput Screening of our libraries of ~200,000 compounds.

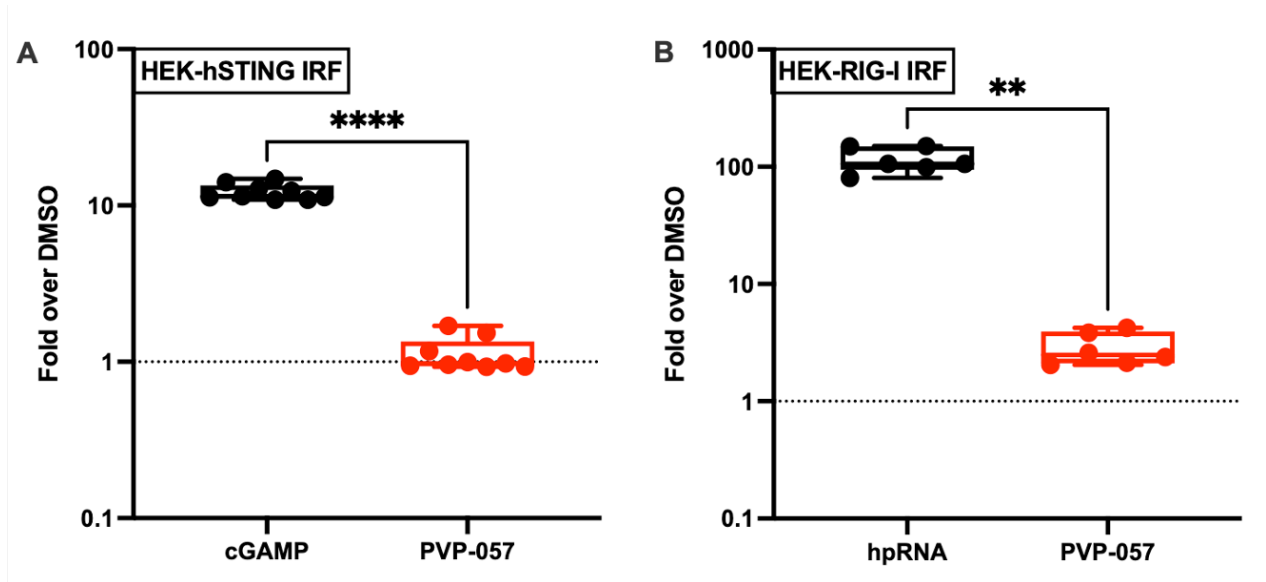


Supplementary Figure 1: PVP-057 promoted maturation of human monocyte-derived dendritic cells as measured by upregulation of co-stimulatory molecules, HLA-DR, and induction of IFN β . Adult monocyte-derived dendritic cells (MoDCs) were stimulated with 6.25, 12.5, or 25 μ M PVP-057 (red) for 24 hours with untreated conditions (grey) used as a negative control. Quantitative expression levels, as measured through median fluorescence intensity (MFI), are visualized for **(A)** CD80, **(B)** CD86, and **(C)** HLA-DR. **(D)** IFN β was quantified via ELISA. Each dot represents a biological donor. N = 6 per condition. Two-sided Mann-Whitney U tests were applied. ^{NS} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$. **(E-F)** Adult monocyte-derived dendritic cells (MoDCs) were stimulated with 12.5, 25, or 50 μ M PVP-057, 25 μ g/mL poly(I:C), or DMSO for

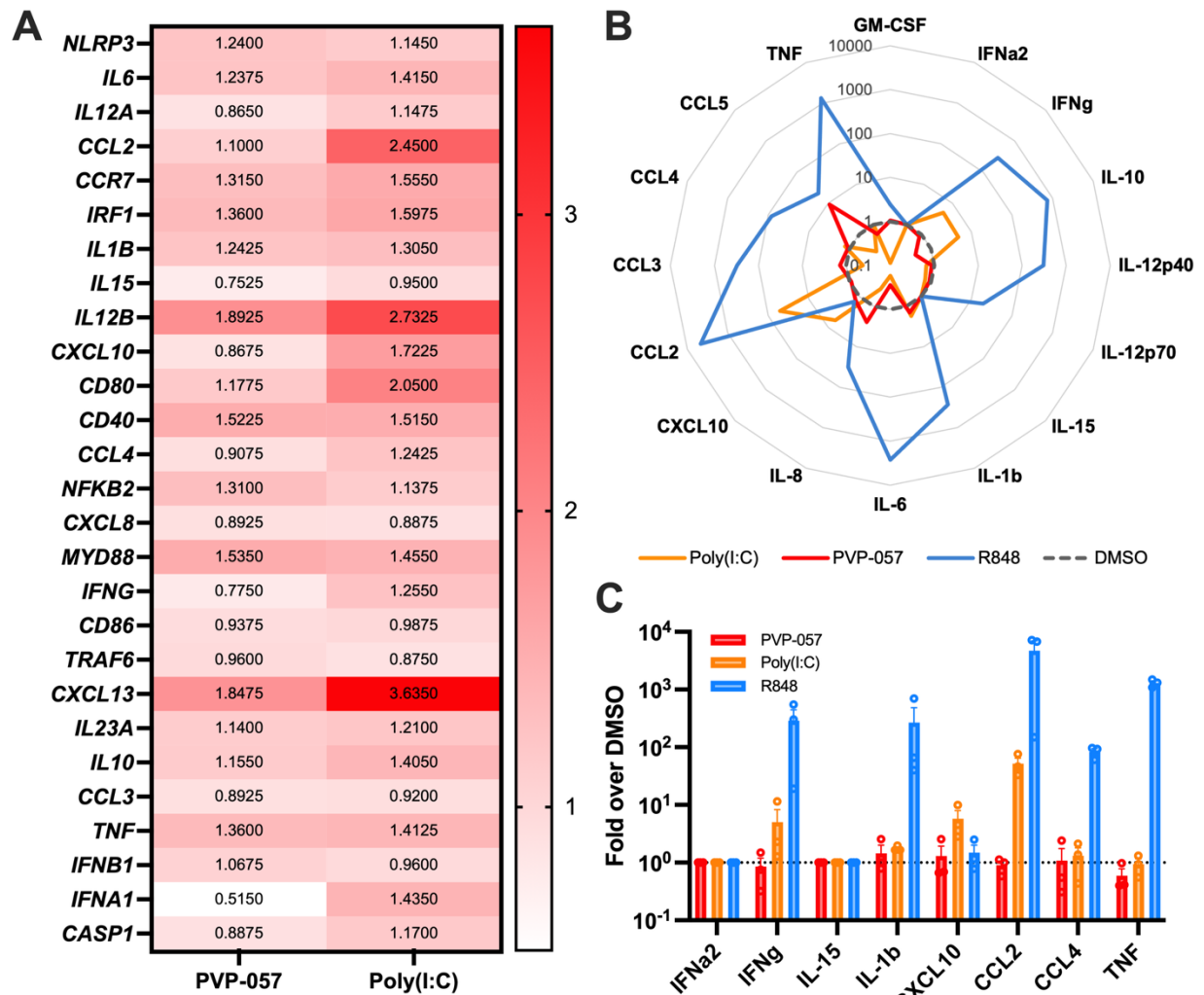
17 18 hrs. Quantitative expression levels, as measured through median fluorescence intensity (MFI),
18 are visualized for **(E)** CCR7 and **(F)** CD40 as fold over DMSO-stimulated group. Each dot
19 represents a biological donor. N = 3 per condition. Source data are provided as a Source Data file.
20



Supplementary Figure 2: HEK-Blue hTLR3 cell line displays hTLR3 selectivity. HEK-Blue hTLR3 cells and their parental HEK-Blue Null1 cells (InvivoGen) were stimulated at 100,000 cells/well. Supernatant was collected 22 hours post-stimulation and NF-kB was quantified via Quanti-Blue detection assay. **(A)** NF-kB induction by 5% (v/v) DMSO, 100 ng/mL NexaVant, 100 ng/mL poly(I:C), 100 μ M R848, or 100 ng/mL TNF. **(B)** NF-kB induction by a dose-titration of poly(I:C) and NexaVant. N = 2 technical replicates per group, except for DMSO, R848, and TNF (N = 4). Source data are provided as a Source Data file.

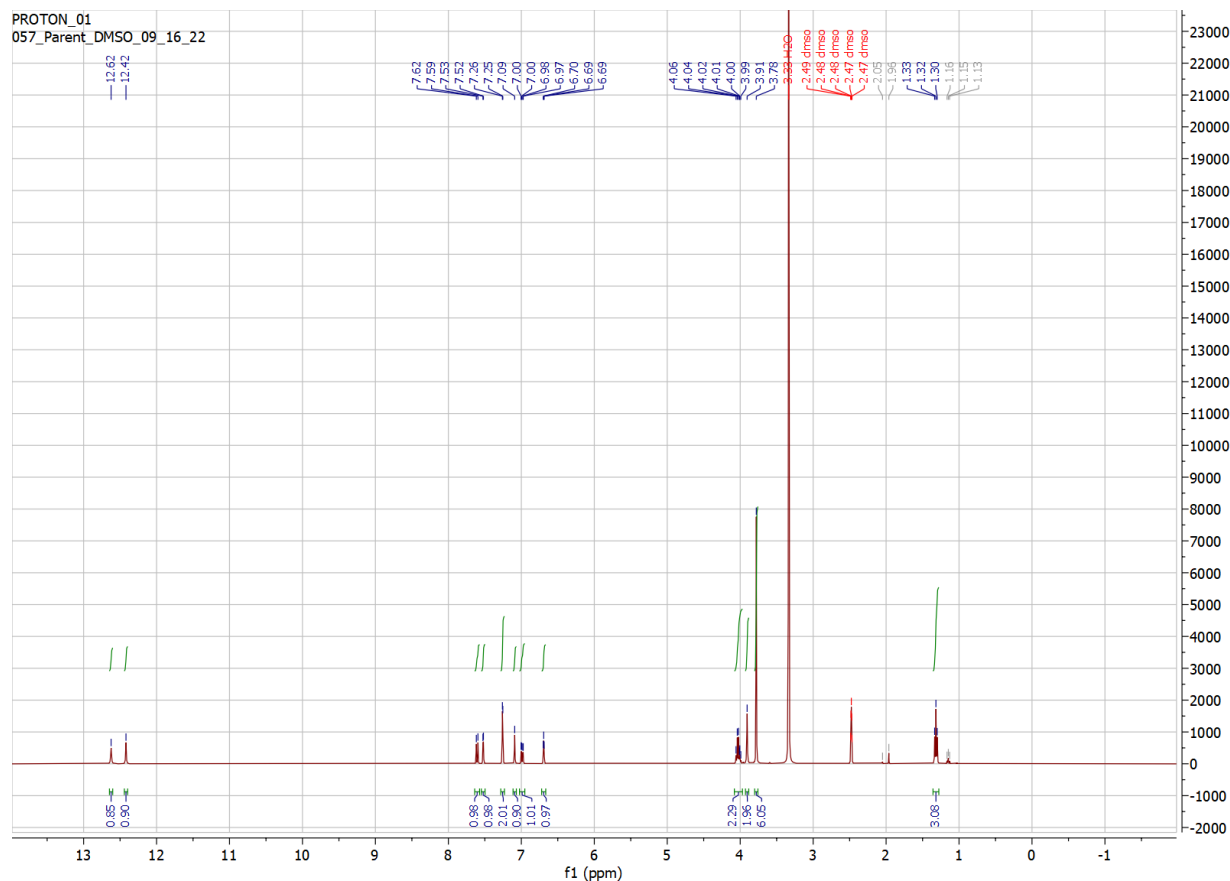


Supplementary Figure 3: PVP-057 demonstrates little to no activation of hSTING or hRIG-I in vitro. (A) HEK-hSTING-overexpressing and (B) HEK-hRIG-I-overexpressing reporter cells were stimulated with 25 μ M PVP-057 and their respective positive controls: 25 μ M cGAMP for STING and 1 μ g/mL hpRNA for RIG-I. Results are shown as fold induction of IRF over the negative control, 1% DMSO (dashed line). N = 9 (A) or 6 (B) technical replicates per condition, combined across two independent experiments. Box plots depict median and interquartile range (IQR), with whiskers extending to minimum and maximum data points. Two-sided Mann-Whitney U tests were applied. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Source data are provided as a Source Data file.



Supplementary Figure 4: Activation of human peripheral blood mononuclear cells. (A) Adult human PBMCs (N = 4 biological replicates) were stimulated for 4 hours with 0.3% DMSO, 33 μ M PVP-057, or 1 μ g/mL poly(I:C). Gene expression was quantified via qPCR and is displayed as fold change over DMSO. (B-C) Adult human PBMCs (N = 3 biological replicates) were stimulated with 25 μ M PVP-057 and 25 μ g/mL poly(I:C) for 18 hours with a DMSO-stimulated group (vehicle) used as a negative control. Cytokine and chemokine concentrations were measured via multiplex assay and are graphed as mean fold over DMSO. Error bars represent SEM. Source data are provided as a Source Data file.

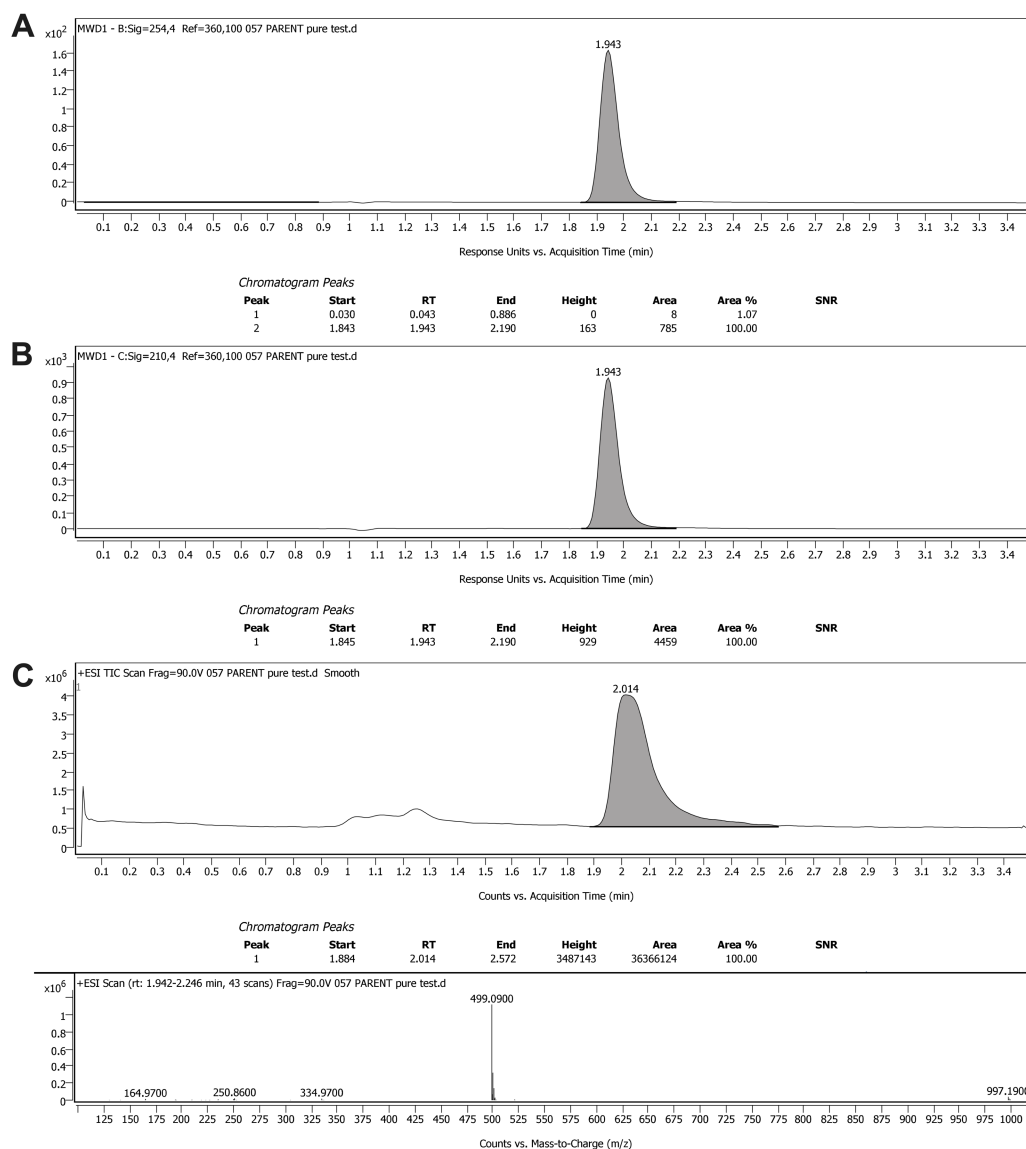
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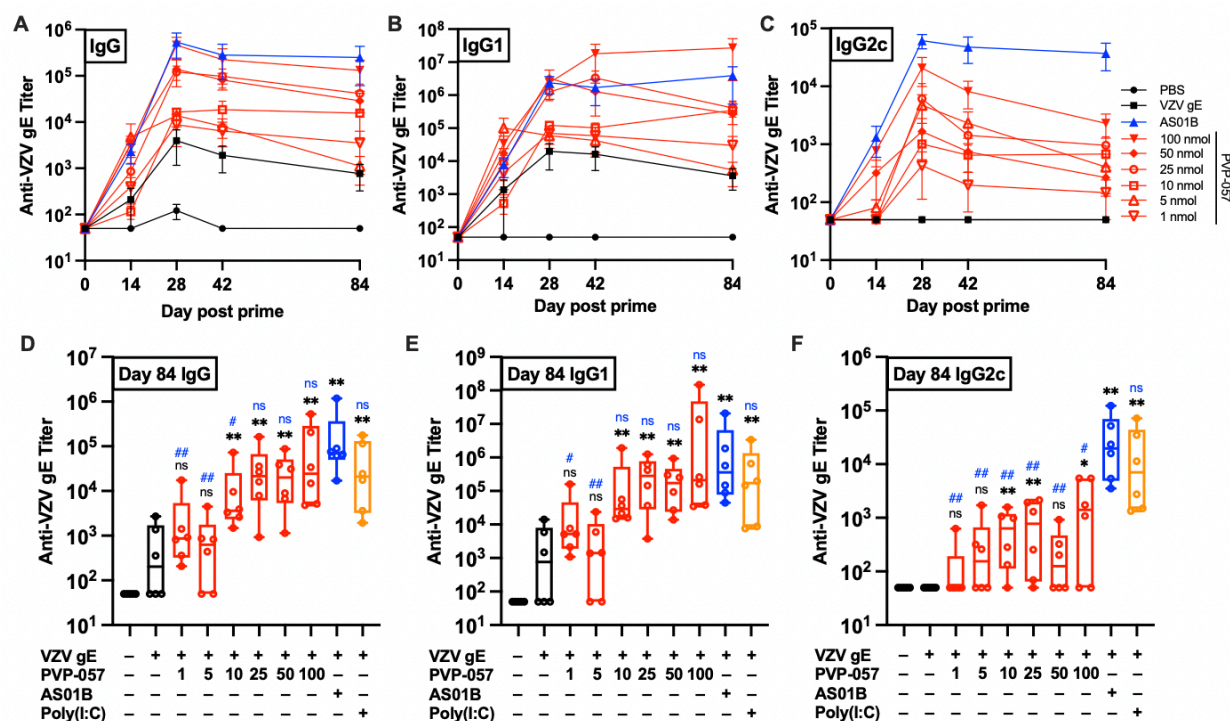
51

52 **Supplementary Figure 5: ^1H Nuclear Magnetic Resonance Spectroscopy of PVP-057**53 **demonstrated high levels of purity via the synthesis process. ^1H -NMR (400 MHz, DMSO) δ** 54 **12.62 (s, 1H), 12.42 (s, 1H), 7.61 (d, J = 8.8 Hz, 1H), 7.52 (d, J = 2.5 Hz, 1H), 7.26 (d, J = 2.3 Hz,**55 **2H), 7.09 (s, 1H), 6.99 (dd, J = 8.8, 2.5 Hz, 1H), 6.69 (t, J = 2.2 Hz, 1H), 4.03 (q, J = 7.0 Hz, 2H),**56 **3.91 (s, 2H), 3.78 (s, 6H), 1.32 (t, J = 7.0 Hz, 3H).**

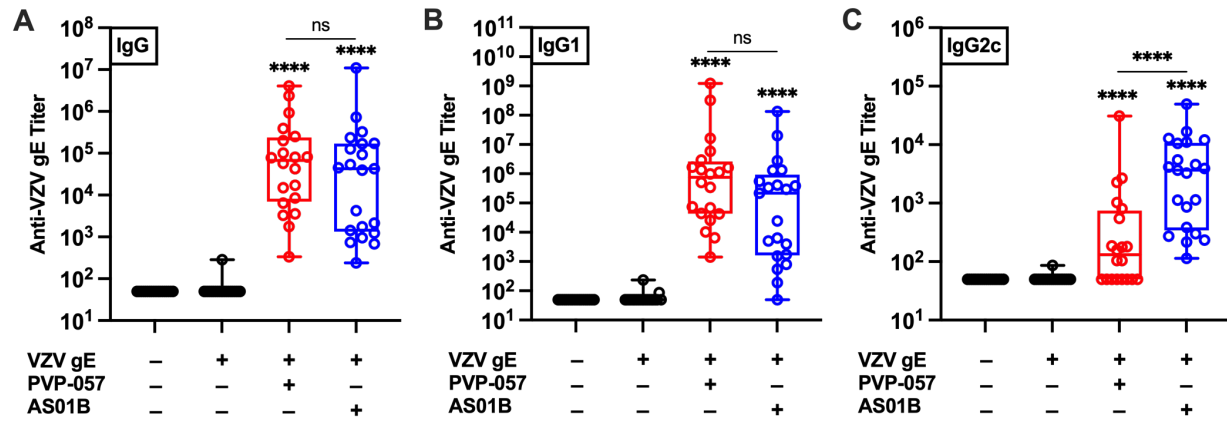
57



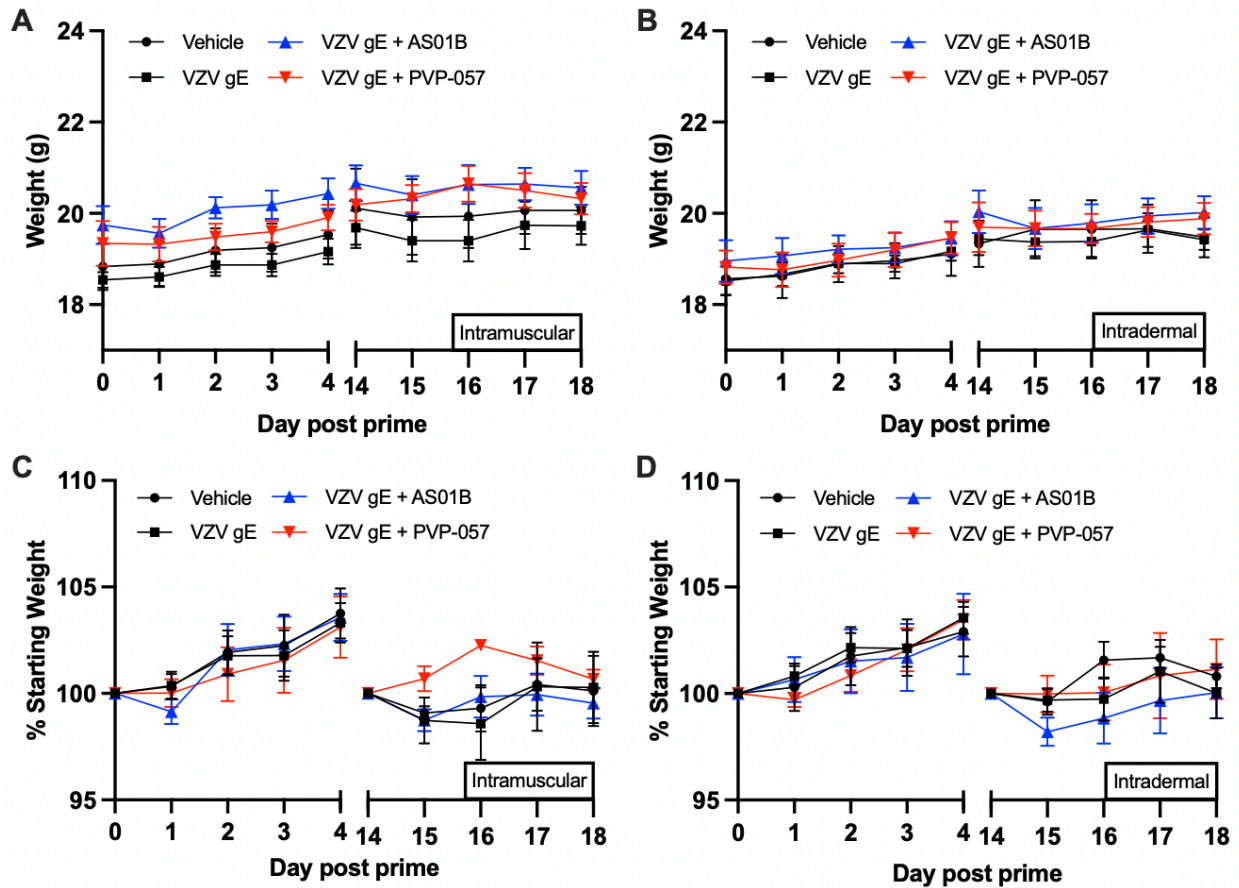
Supplementary Figure 6: Liquid Chromatography-Mass Spectrometry analysis of compound PVP-057. Agilent UPLC-MS Ultivo, LC column Poroshell 2.7 μ m. Solvent system: LC-MS grade H₂O + 0.1% FA / MeCN **(A)** 254 nm UV spectra: peak 1 at 0.030 min, 1.07%. PVP-057 at 1.943 min, 98.93%. **(B)** 210 nm UV spectra: PVP-057 at 1.943 min, 100%. **(C)** Qualitative MS analysis Total Ion Count (TIC): PVP-057 at 2.014 min, 98%; 498.1 m/z.



Supplementary Figure 7: In vivo potency of PVP-057. (A-C) Adult C57BL6/J mice were immunized ID with PBS, 0.5 μ g VZV gE alone, or VZV gE admixed with AS01B (1/100th human dose), 10 μ g poly(I:C), or PVP-057 at 1, 5, 10, 25, 50, or 100 nmol on Day 0 and Day 14. Serum samples were collected on Days 14, 28, 42, and 84 post-prime immunization. Longitudinal anti-VZV gE IgG (A) IgG1 (B), and IgG2c (C) titers were assessed. Data is presented as mean $\pm$ SEM. (D-F) Titers shown for (D) IgG, (E) IgG1, and (F) IgG2c on Day 84. N = 6 biological replicates per group, except for PBS group (N = 5). Significance against VZV gE alone is shown with asterisks. Box plots depict median and interquartile range (IQR), with whiskers extending to minimum and maximum data points. One-sided Mann-Whitney U tests were applied for comparisons against VZV gE alone. ^{ns} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Significance against AS01B is shown with hash signs. Two-sided Mann-Whitney U tests were applied for comparisons against AS01B. ^{ns} $p > 0.05$, # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$. Source data are provided as a Source Data file.

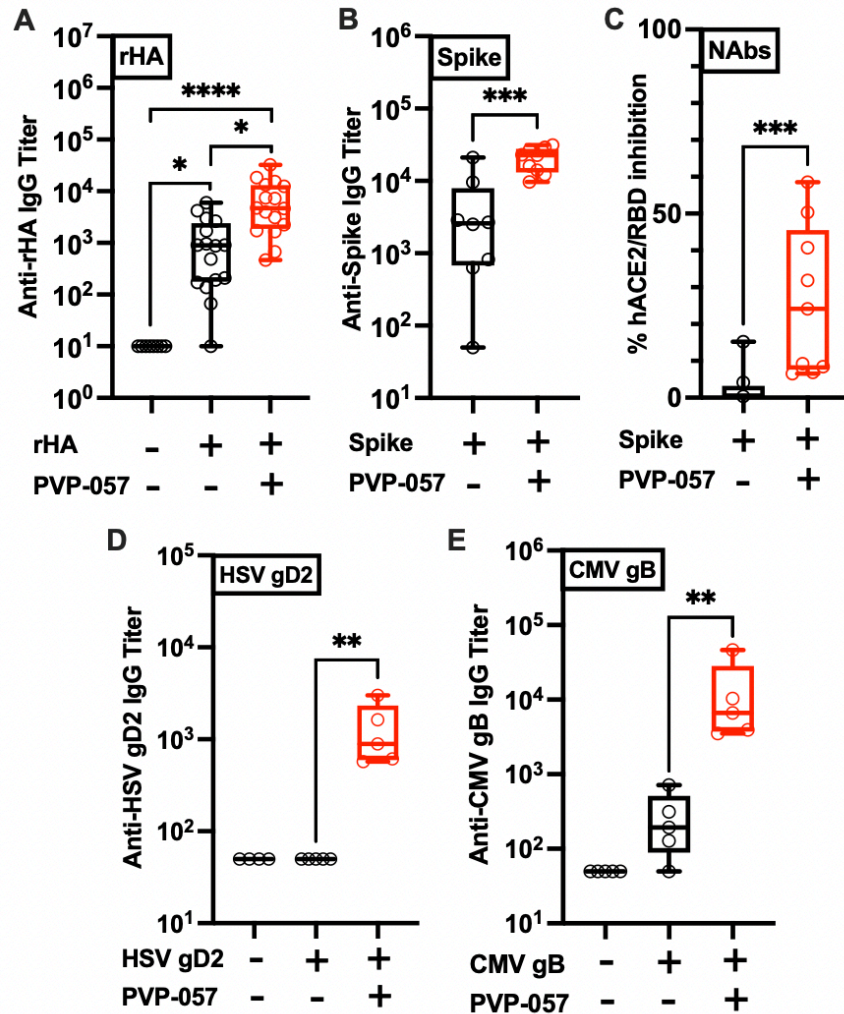


Supplementary Figure 8: Serological consistency of PVP-057. (A-C) Adult C57BL6/J mice were immunized ID on Day 0 and boosted on Day 14 with PBS, 0.5 μ g VZV gE alone, or VZV gE admixed with PVP-057 (100 nmol) or AS01B (1/100th human dose). Serum samples were collected on Day 42. Anti-VZV gE IgG (A) IgG1 (B), and IgG2c (C) titers were assessed. N = 18-21 biological replicates per group across three separate experiments. Box plots depict median and interquartile range (IQR), with whiskers extending to minimum and maximum data points. Significance against VZV gE alone is denoted above individual groups, for which one-sided Mann-Whitney U tests were applied. Significance between PVP-057 and AS01B-adjuvanted groups is shown, for which two-sided Mann-Whitney U tests were applied. ^{NS} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Source data are provided as a Source Data file.



Supplementary Figure 9: Mice immunized with PVP-057 demonstrate normal weight gain.

Adult 6 to 8-week old C57BL/6 mice were immunized IM or ID on Day 0 and boosted on Day 14 with 0.5 μ g VZV gE $\pm$ AS01B (1/100th human dose) or 100 nmol PVP-057 admixed. Mice were weighed on Day 0 (pre-immunization) through Day 4, and Day 14 through Day 18. Weight change over time is shown for mice immunized IM (A) and ID (B) and displayed as percent of starting weight for mice immunized IM (C) and ID (D). N = 7 biological replicates per group, except for PBS and VZV gE alone groups (N = 6). Data graphed as mean $\pm$ SEM. Source data are provided as a Source Data file.

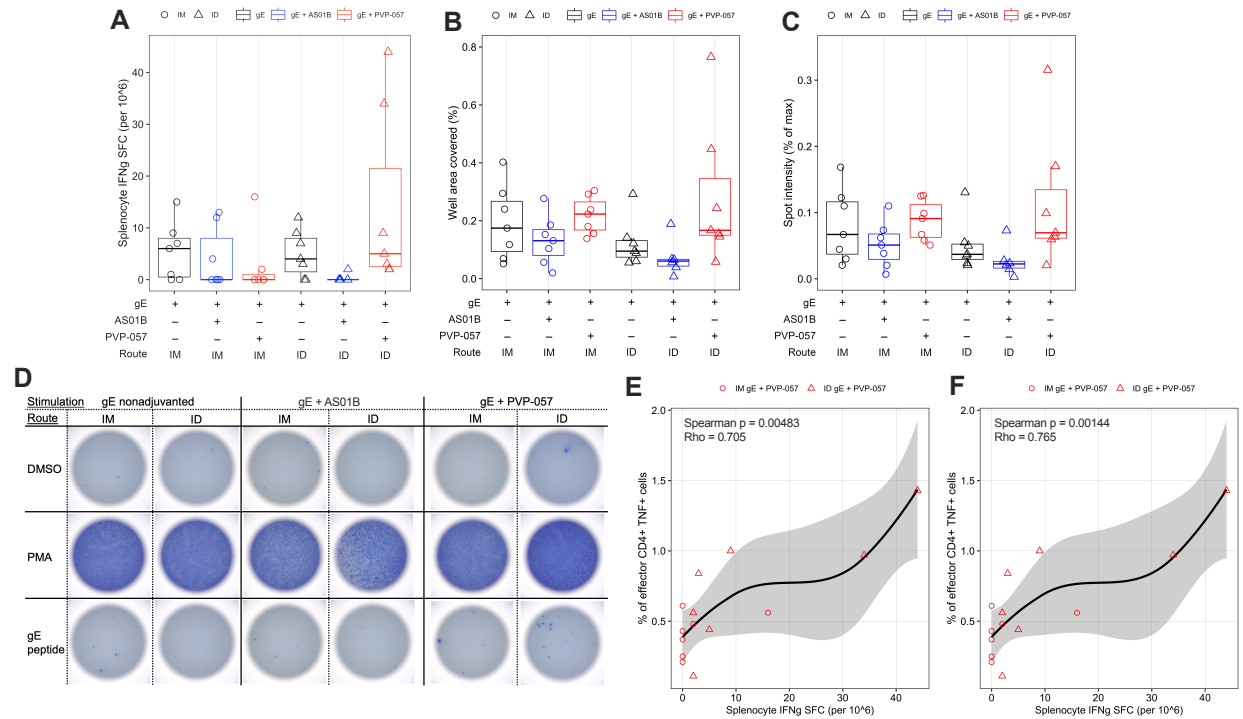


Supplementary Figure 10: PVP-057 demonstrated robust adjuvanticity towards viral vaccinal antigens in vivo. 6 to 8-week-old BALB/c mice were immunized IM with PBS, rHA alone, or rHA admixed with 100 nmol of PVP-057. Serum samples were collected 14 days after initial immunizations. **(A)** Anti-rHA IgG titers were assessed. N = 16 biological replicates per group except for PBS group (N = 8). **(B-C)** 6 to 8-week-old BALB/c mice were immunized IM with SARS-CoV-2 spike protein with or without 100 nmol of PVP-057. Mice were immunized on Day 0 and boosted on Day 14. Serum samples were collected 28 days after the initial immunizations. **(B)** Anti-SARS-CoV-2 spike IgG titers and **(C)** hACE2-RBD inhibition, via quantification of neutralizing antibodies (NAbs), was assessed. N = 8 (Spike alone) or 10 (PVP-

057 adjuvanted) biological replicates per group. **(D)** 6 to 8-week-old C56BL/6 mice were immunized IM with PBS, 5 μ g HSV gD2, or HSV gD2 admixed with 100 nmol of PVP-057. Mice were immunized on Day 0 and boosted on Day 14, and serum samples were collected on Day 42. Anti-HSV gD2 IgG titers were assessed. N = 5 biological replicates per group except for PBS group (N = 4). **(E)** 6 to 8-week-old C56BL/6 mice were immunized IM with PBS, 5 μ g CMV gB, or CMV gB admixed with 100 nmol of PVP-057. Mice were immunized on Day 0 and boosted on Day 14, and serum samples were collected on Day 42. Anti-CMV gB IgG titers were assessed. N = 5 biological replicates per group. Box plots depict median and interquartile range (IQR), with whiskers extending to minimum and maximum data points. One-sided Mann-Whitney U tests or Kruskal-Wallis test with post-hoc Dunn's test were applied according to the number of groups.^{NS} $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$. Source data are provided as a Source Data file.

136 in murine splenic effector T cell compartments 6 hrs post-simulations using 1 µg/mL of VZV gE
137 peptides.

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Supplementary Figure 12: ID-administered PVP-057 significantly increased the number of IFN γ SFCs. Mice were immunized and evaluated as in Figure 7. Splenocytes stimulated with 1 μ g/mL VZV gE peptide were evaluated by ELISpot for spot forming cells (SFC), with baseline subtraction of DMSO-stimulated controls. Immunospot-reported (A) SFC, (B) well area covered (%) and (C) spot intensity (% of maximum) were determined. (D) Representative images of ELISpot wells for VZV gE non-adjuvanted, AS01B-adjuvanted, or PVP-057-adjuvanted groups, either IM- or ID-vaccinated, are presented. The topmost row represents vehicle control (DMSO) baseline stimulation of splenocytes, followed by the middle row of a positive control Phorbol Myristate Acetate (PMA) stimulation, and the bottom row of 1 μ g/ml VZV gE peptide-stimulation. A correlation analysis of flow cytometry-evaluated IL-2 (E) or TNF (F) responsiveness (y-axis) versus IFN γ ELISpot (x-axis) displayed a significant positive correlation, with the 95% confidence interval displayed in grey. Box plots depict median and interquartile range (IQR), with whiskers extending to minimum and maximum data points. Following a Shapiro-Wilk test

for normality, statistical significance was determined by a Kruskal-Wallis test with post-hoc
Dunn's test (A-C). For correlation evaluations, Spearman's correlation was employed (E-F). N =
7 biological replicates per group, except for ID gE + AS01B group (N = 6). ^{NS} $p > 0.05$, non-
significant unless otherwise noted. Source data are provided as a Source Data file.

Target	Clone	Fluorochrome	Vendor	Identifier	Titer	Staining step
CD3	17A2	BUV496	BD	741117	1:80	Surface
CD4	RM4-5	APC Cy7	BD	565650	1:80	Surface
CD8	53-6.7	BUV395	BD	563786	1:80	Surface
CD19	1D3	PE CF594	BD	562329	1:320	Surface
CD44	IM7	RB744	BD	757245	1:80	Surface
CD45	30-F11	RB780	BD	569151	1:160	Surface
CD45R	RA3-6B2	BV605	BioLegend	103243	1:40	Surface
CD62L	MEL-14	BUV737	BD	612833	1:160	Surface
IFN γ	XMG1.2	AF488	BioLegend	505813	1:160	ICS
IL-2	JES6-5H4	PE	BioLegend	503808	1:160	ICS
IL-4	11B11	BV421	BioLegend	504119	1:80	ICS
IL-5	TRFK5	BV421	BioLegend	504311	1:160	ICS
IL-17A	TC11-18H10	R718	BD	567098	1:80	ICS
NK1.1	PK136	BV711	BioLegend	108745	1:80	Surface
TCR $\gamma\delta$	GL3	BUV563	BD	748993	1:80	Surface
TNF	MP6-XT22	PE Cy7	BioLegend	506324	1:160	ICS
Viability	---	L/D Aqua	Invitrogen	L34966	1:500	Surface

Supplementary Table 2. Table for flow antibodies used in the optimized 17-parameter panel according to OMIP-111⁶⁷. Titer represents the final dilution factors. The last column indicates the targeting staining step where the antibodies were used.